STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

RESEARCH PROBLEMS STILL FACING THE STAIN COMMISSION

The Committee on Standardization of Biological Stains, which after a year's activity developed into the present Biological Stain Commission, was organized by the National Research Council in December 1921. The work on Stain Standardization has, therefore, been in progress for ten years. During that time the plan of certifying stains has been developed and about fifty of the most commonly used stains put on the certification basis. It is now frequently asked if the work of the Commission has not become wholly routine—the testing of samples submitted to see if they meet the requirements for certification; and if the work is not entirely routine after ten years of effort to establish standards, why isn't it?

There have been various forces operating to keep the work of the Commission from becoming mere routine. Important among these has been the fact that its objects have been continually changing—constantly broadening. The problem before the original committee of the National Research Council was to see if American dyes could be made the equal of those previously imported from Europe. It was quickly discovered, however, that in the case of the most commonly used stains this had already been accomplished. In the first place, no true standardization of stains had ever been effected in Europe; different batches of the same dye from a single manufacturer could vary tremendously, and some were entirely unsatisfactory. In the second place, there were dyes manufactured in America even at that time which were highly satisfactory for biological use; and altho there were exceptions—exceptions that had given the American-made stains a bad name—these could in nearly all cases be matched by equally poor lots imported from Europe. Accordingly the real problem which the committee faced was one of education: first, education of the users as to the high quality of many American products, with information as to which dealers handled them; and second, education of the manufacturers as to the needs of the biologists, expressed in terms that the former could understand.

The first of these tasks, education of the biologists, was under-

taken by a series of articles in certain scientific journals. Response was soon evident from the increased interest in American stains that began to be shown on all sides.

The second task was more difficult; but it was equally necessary, for until the manufacturer knew in terms of dye production just what the biologist wanted he could not be expected to duplicate an especially good batch of dye when the first lot was exhausted. Before this could be accomplished much education of the committee itself was required. We were, like most other biologists at that time, deplorably ignorant as to the chemistry of stains. If we did not like a stain, about all we could say was that it did not work; and that was not much help to the manufacturer. The usual attempt on the part of a biologist to make his complaint more intelligible to the manufacturer was to say that the American dye was not pure enough. Stain manufacturers usually tried to be accommodating on receipt of such complaints, even tho they were morally certain that no imported sample had ever been freer from impurities than their own product; but picture their discouragement when after an expensive process of purification they found the result less satisfactory to the biologist than the original lot. Such was frequently the case; for it was rare that the dissatisfaction of the biologist was due to too low dye-content. The committee promptly realized this; but it was not until we had given the subject much study that we were able to tell the manufacturer what might cause the dissatisfaction in any particular instance. In other words the committee soon found it necessary to train itself to act as an interpreter between biologist and manufacturer.

A good illustration as to the obscure nature of some of the difficulties of that time and our own inability to explain them is furnished by the dye, rose Bengal, thru which our attention first was called to the need of standardization of stains. This dye had been used in the Chairman's laboratory since 1917; but when the first lot of American-made rose Bengal was employed, about two years later, the staining was altogether too weak. The matter was referred to R. T. Will of Rochester, the last lot of dye having been purchased from the Will Corporation. By a lucky chance he succeeded in supplying us with a satisfactory batch, and did the far greater service of calling our attention at the same time to the need of standardization in the entire field of biological stains. Not till a few years ago, however, was the probable cause of the unsatisfactory results with the first domestic batch determined: namely that the pure sodium salt of rose Bengal never stains deeply in the technic in

question, but that the presence of small amounts of calcium or magnesium impurities convert a certain percentage of the dye into the salt of the heavier metal which stains more deeply. Had this been suspected at first, the staining technic could have been put on a scientific basis then instead of nearly ten years later.

Thus each stain was found to present its own problems; and as they were solved, one by one, it began to be appreciated that we were getting a knowledge of the behavior of dyes in staining that was new to scientists as well as manufacturers, both in this country and abroad. With this knowledge came the realization that the true aim of the Commission should not be to secure the production of domestic stains equal to those from Germany or any other country, but to put the production and use of biological stains on a scientific basis, and incidentally to secure the production of stains of a better quality than ever before. It was a vision looking far into the future, a vision that has by no means yet been realized. Such an undertaking had never been attempted before; and it was unlikely that conditions would occur again as favorable for its accomplishment as existed in the United States five years after the war. We had a body of biologists organized for testing stains and enthusiastic in the matter of improving the supply; we had manufacturers willing and able to coöperate; and finally, thanks to the Chemical Foundation, we had funds available to finance the work until it had either become self-supporting or had proved its necessity enough to appeal to other sources of support.

Once it was decided to aim for a truly scientific standardization of stains, and when at the same time it was realized what a hit-or-miss matter biological staining had always been, it became plain that the work would never within our lifetime develop into pure routine. This becomes clearly evident when one considers that new uses of stains are constantly being devised, which often upset past efforts in the direction of standardization. A few illustrations as to what has been done with certain stains, and what still remains to be done before their complete standardization can be accomplished will make this point clear.

There are some stains, to be sure, which we consider at present to have been given a satisfactory degree of standardization. Even with these one cannot be sure, for some new use for one of them may be proposed at any time, in which some lots are good, others unsatisfactory—and then for this particular dye the whole problem will be opened up again. We regard methylene blue as being on a very satisfactory basis; and it has been ever since it was learned

that the medicinal grade of this dye, if sufficiently free from mineral impurity and if prepared in such a way as to permit a certain amount of oxidation into lower homologs, would serve for any of the biological uses to which it is now put. After studying the eosin group of dyes for several years, we consider these dyes, as now offered to biologists by American concerns, to have a high degree of reliability; for here the trouble was not so much lack of standardization as actual mislabelling of the imported stains available before the war, so that the biologist did not know what he had really been using. Light green at one time proved puzzling, for there are several dyes of this name distinguished from one another by various shade designations; and it was natural that the American manufacturers at first would sometimes supply the wrong one. When it was finally learned that the particular green dye desired by biologists is light green SF yellowish, it looked as tho the problem presented by this dye were solved. Recently, however, the new dye, fast green FCF, has been called to our attention as a splendid substitute for light green, as it is permanent whereas the latter fades rapidly; so now the problem in connection with this dye is to get biologists acquainted with the new and better substitute for it. Even the natural dyes prove capable of standardization. The manufacturers of hematoxylin, for example, have, in coöperation with certain members of the Commission, worked out a method of manufacture which gives a very uniform product that has not yet called forth any serious complaints from its users. So with various other stains—for the present they may be considered as standardized; but some biologist may at any time propose some new technic for one of them which will entirely upset the present scheme of things.

Many other dyes, however, present problems that are acutely before us at present. Take safranin, for instance. It is true, to be sure, that some of the original problems presented by this stain have been solved. At the beginning, for instance, it was not known which of the various dyes and shades of dyes going under the name of safranins were desired by histologists and cytologists. This problem was not very baffling, however, as it was quickly learned that safranin O, defined as having its color absorption maximum at 515 mμ, meets the shade requirements of biologists. These shade requirements, moreover, are rather exact, for the dye is frequently employed in the Flemming triple staining technic, in which it must contrast both with gentian violet and orange G, so that too blue or too yellow a shade is unsatisfactory. Somewhat puzzling at first were the orders received by stain companies for alcohol-soluble or for water-

soluble safranin, inasmuch as all safranins are almost as soluble in the one as in the other. It is not yet known, to be sure, what may have been offered under these two names before the war; but the present German products sold in this country under these two designations seem to be identical so far as chemical and optical tests can show. Both are equally soluble in alcohol and in water. There is absolutely no reason, apparently, for recognizing the two types.

Were this the whole question, safranin might be considered out of the way as a problem. But puzzling complaints still come in concerning samples of this stain. Some samples seem to stain more rapidly than others or to decolorize more slowly. Two samples thus differing may be identical so far as can be told by any chemical or physical tests at present available; they may have both been prepared by the same manufacturer by identical methods of purification from a single lot of crude safranin. But cytologists insist there is a difference. How real the difference is, and what causes it, if it is real, are questions we cannot yet answer. We surmise that the difference is caused by impurities present. The problem may not be solved for years, because manufacturers seldom submit samples of this stain (which is not used in large quantity); and the technic by which it is used is so complicated that reports from different users are often conflicting. We know that samples of this dye now on the market are much more reliable than they were formerly; but from a scientific standpoint the stain is not standardized.

Equally unsolved is the problem of basic fuchsin. The chief trouble with this dye is that it is used for many widely divergent purposes, and in this country we are rather opposed to the plan of putting on the market various grades of the same dye, each designed for a different use. More basic fuchsin is used by bacteriologists than by all other biologists together; but even bacteriologists use it for two distinct purposes. It must, first and foremost, be a good bacterial stain and must serve for bringing out the "acid-fast" properties of the tubercle organism. The diagnosis based on this staining technic is so important that no fuchsin can be considered satisfactory that fails for this purpose. Secondly, bacteriologists use the dye in the Endo medium for the recognition of the typhoid organism. The Endo medium is apparently based upon Schiff's reaction. Basic fuchsin, decolorized with a sulfite or with sulfurous acid, is known as Schiff's reagent and has the property of reacting to the presence of aldehyde by the restoration of its red color. This dye, thus decolorized, and added to a lactose agar, becomes the Endo

medium; growing on it, the typhoid organism produces little or no change in color, the colon organism, however, restores the red color with the appearance of a deep metallic sheen on the surface, while the closely related aerogenes type produces a pink color without the metallic sheen. That is, they do if the right basic fuchsin be used. Not all basic fuchsins, however, behave properly in this reaction; and the bacteriologist does not like to use different fuchsins for this medium and for staining slides. On the surface, the behavior of the dye in this test is a simple Schiff's reaction; but there is some question as to whether this reaction explains everything. It is certainly true that samples of the dye that behave well as Schiff's reagent when tested by observing their ability to detect aldehyde, do not necessarily give a clear-cut Endo reaction.

For years the manufacturers in this country have been striving, with the help of the few suggestions we have been able to offer, to produce consistently a basic fuchsin satisfactory for the two purposes described above. Occasionally they hit the combination; but apparently only by chance, for they cannot consistently duplicate a satisfactory batch. We were, nevertheless, becoming encouraged due to their more frequent success in this matter during recent years; but recently one of those upsetting new procedures was proposed that has caused unexpected complications. A few years ago some Germans proposed the use of Schiff's reagent as a microchemical test for thymonucleic acid in cell nuclei. Recently this Feulgen reaction—as it is called from its author—has been taken up by cytologists in this country, as a chemical test for chromatin and is coming into considerable prominence. The chief drawback in the technic is that not all fuchsins are equally satisfactory in the procedure, and the cytologist is puzzled as to whether his poor results are due to faulty technic or to an unsatisfactory dye. Theoretically the same basic fuchsin that behaves in the Endo medium should work in the Feulgen reaction; in practice, however, that is not the case. A few samples of basic fuchsin have been found that work in this technic and are also satisfactory for the two bacteriological procedures. Unfortunately, however, most of them are batches no longer on the market; and worse yet, apparently no one knows why they are more satisfactory than others.

The fuchsin problem, therefore, is still baffling. There seems to be no correlation between the behavior of this dye and the proportion of rosanilin and pararosanilin (the two main constituents of basic fuchsins) that are present; nor can it be shown to have any distinct relation to whether the dye is supplied as the chloride or the

acetate—the two most common salts of this dye on the market. In fact, here again, as in the case of safranin, we are beginning to explain the difference on the basis of impurities present; only in this case we suspect an impurity which obscures the chemical reaction involved in the Endo and the Feulgen technics. The nature of the impurity and how to exclude it, however, are still unanswered questions.

If some of these problems with simple dyes are still baffling, how much more so are those involving the compound dyes, the blood stains! Blood stains, as is well known, are ordinarily compounds of eosin with polychrome methylene blue. The latter, moreover, is no pure dye, but is a mixture of methylene blue with various oxidation products (the azures, etc.) in undetermined quantity. Such being the case, it is natural that blood stains vary in their composition and behavior. Attempts have frequently been made to develop a synthetic blood stain, which would be of known and constant composition, but so far they have not been entirely successful.

To make the problem still more baffling, blood stains are very difficult to judge on the basis of their performance. A blood stain may appear very good on casual inspection of a slide stained with it, and yet prove unsatisfactory if the slide is given careful study. The most common defect of a blood stain is in failing to bring out the proper difference in color between the granules of the neutrophiles and of the acidophiles; and considering that the latter type of cells rarely constitutes more than two percent of the leucocytes present in normal blood, a long search is often necessary before the quality of a stain can be properly judged. Another complication arises from the fact that with a little manipulation nearly any fairly good Wright stain can be made to show up this difference in a satisfactory way. As a result, to the clever technician there are very few unsatisfactory blood stains; while among the less skilled technicians, examining the same two samples of Wright stain, some may find the first more satisfactory, others the second. Even this does not complete the list of difficulties; for some samples of Wright stain deteriorate in solution, while others tend to improve after the solution has been standing a few weeks. A blood stain that yields a fairly permanent solution is naturally much to be desired; but the tests necessary to determine its permanency are, of course, time-consuming. All this makes the accurate judging of blood stains a difficult matter; and even after careful study and conscientious discrimination, we are often afraid we have, on the one hand, approved a stain that will be unsatisfactory in the hands of the average technician, or on the other,

rejected a sample fully as good as some that we have passed without question.

Blood stains present the most difficult problem of all at the present time—a problem that probably will not be solved until someone has devised a reliable and economical method of making a synthetic Wright stain.

With such problems as this still before us, the work of the Stain Commission shows no sign of becoming purely routine. And no one can ever tell when a new problem will develop. Thus, we thought until about a year ago that methyl green was on a satisfactory basis. But recently Miss Scudder[1] has proposed an apparently important combination of the Gram and Pappenheim stains, in which the ordinary grade of methyl green will not work. A specially prepared sample furnished by one manufacturer was entirely satisfactory; but duplication of this sample (as judged by performance) is proving quite difficult. It can be said without hesitation that problems related to dyes can safely be expected to furnish the Stain Commission with research work for years to come.—H. J. Conn.

[1]Scudder, Sara A. A differential stain favorable to the diagnosis of Neisserian infection. Stain Techn., **6**, 99-105. 1931.

FEULGEN'S REACTION AND SOME OF ITS APPLICATIONS FOR BOTANICAL MATERIAL

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ABSTRACT.—**A brief description of Feulgen's reaction is given. Normally treated sections of apple buds, roots of *Pandanus*, *Narcissus*, cabbage infested with *Plasmodiophora brassicae*, etc., algae, intestine, kidney and testes, and smears of yeasts were studied. Acids and alkalies were used to alter the chemical composition of chromatin and in thus doing a check-up for chromatin was feasible. Nucleoli never reacted, except when sections were specially treated. Lignin, suberin and cutin were observed to react in normally treated tissue no matter which of the several fixatives was used, if any. A procedure for their detection, with a possible explanation for the reaction, is offered.**

Seven strains of yeasts were tested. All showed a positive reaction with a more or less fusiform purplish nucleus. Unhydrolyzed yeasts showed a number of reddish granules under the action of the fuchsin sulfurous acid. In contrast to Feulgen's reaction, this color (red, not purple) is apparently due to simple oxidation and not to the presence of aldehydes.

THE PROCEDURE

Feulgen's reaction is not yet generally used in America for cytological or histological investigations. This is particularly true in regard to botanical material, undoubtedly because of the general impression that plant nuclei do not give this reaction. It proves, however, to have distinct value in botanical work.

Disregarding the value of Feulgen's reaction as a chemical test for chromatin, the clarity of its pictures, where nothing except the chromatin is stained, offers tremendous advantages over the usual so-called nuclear stains, which give little differentiation between chromatin, nucleoli, and various extra-nuclear bodies. A description of the theoretical basis of the reaction as well as the method itself may be found in Krause (1927).

Briefly the procedure is as follows: fix preferably in saturated $HgCl_2$ with 2% acetic acid; or other fixatives may be used, as will be mentioned later. Wash thoroly, varying the time with the size of the object. Run up thru the alcohols, embed, section, and mount. Run sections down to water and treat with 0.5 normal HCl for 2 or more minutes, treat with the same at 60°C for 2 or more minutes, rinse in

the cold HCl, then in water and stain in the fuchsin sulfurous acid for 1 to 3 hours. Prepare this as follows: dissolve 1 g. basic fuchsin by bringing it to boiling in 200 cc. distilled water, cool to 50°C, filter, add 20 cc. of the 0.5 normal HCl, cool to 25°C and add 1 g. sodium bisulfite. Cork well and put in a dark place. The solution should decolorize to a straw color, usually within 24 hours. After staining pass thru three successive baths of 200 cc. distilled water with 10 cc. of 0.5 normal HCl and 10 cc. of 10% sodium bisulfite. Rinse in distilled water, run up thru the alcohols, counterstain, if desired, with any of the usual counterstains, dehydrate, clear and mount.

The kind of fuchsin used for Feulgen's reagent is of great importance, since some fail to decolorize, give a non-specific brownish color, or fail to react at all. Fuchsins of the rosanilin type seem to be best adapted for the purpose. The deepest and most desirable purple in the preparations has been obtained with Grübler's fuchsin f. B.; nevertheless, a fuchsin of the Coleman and Bell Company, certification No. CF-10, is continuously used here, for altho it gives a reaction slightly weaker than Grübler's, it completely decolorizes and does not leave any sediment in the solution, as the latter does. A rosanilin chloride of the National Aniline Company (Lot No. 5110) has also given quite satisfactory results.

Test for Chromatin

Several organisms have been tested for chromatin using the method, both on normally treated tissues, and also following alkalies and acids, which were supposed to alter the chemical properties of chromatin.

Paraffin sections of a Myxomycete, *Plasmodiophora brassicae* in roots of *Brassica oleracea* (cabbage), following Gilson's, Feulgen's and Carnoy-Lebrun's fixatives, showed nuclei and mitotic figures. Sections of an alga (*Cladophora*) fixed in Feulgen's, Gilson's or chromo-acetic mixtures showed nuclei and excellent chromosomes. A diatome (*Navicula*) showed delicately stained nuclei. *Allium* (onion) and *Narcissus*, fixed in Feulgen's, Gilson's, Flemming's or Bouin's solutions, showed a fine, well-defined spireme of the metabolic nucleus and excellent pro-chromosomes and chromosomes. Kidney, testes and intestinal tissue, fixed in either Feulgen's, Bouin's or Zenker's fluids, also gave a clear reaction. The nucleoli showed no reaction in any of these organisms.

While studying sections of several roots and apple buds, it was repeatedly observed that not only chromatin, but also lignin, suberin and cutin gave a positive reaction. A section of the intestine of a

guinea pig fixed in Zenker's fluid showed very definitely stained tracheae, probably remains of carrots or other such food eaten by the animal.

Results obtained from soaking sections in alkalies and acids were as follows: KOH, 2N sol., kidney, Bouin's fixation, pale reaction of nuclei and nucleoli, *Allium*, Flemming's fixation, weak chromatin reaction; Na_2HPO_4, saturated solution, *Brassica* (cabbage) with *Plasmodiophora*, Feulgen's fixation, no chromatin reaction, lignin and cutin positive; acetic acid, 5% sol., *Brassica* with *Plasmodiophora*, Feulgen's fixation, chromatin no reaction, lignin and cutin positive, *Allium*, Flemming's fixation, slight reaction, kidney, Bouin's fixation, weak reaction; oxalic acid, 5% sol., *Brassica* with *Plasmodiophora*, Feulgen's fixation, no chromatin reaction, cutin and lignin positive, *Allium*, Flemming's fixation, and kidney, Bouin's fixation, slight reaction of nuclei and nucleoli; phenol, 5% sol., *Brassica* with *Plasmodiophora*, Feulgen's fixation, chromatin no reaction, lignin and cutin positive, *Allium*, Flemming's and kidney Bouin's fixation, slight reaction of chromatin and nucleoli.

These reactions, or their absence, indicate how precise Feulgen's test is. Thus in *Plasmodiophora*, with relatively small nuclei and less chromatin than in other organisms tested, the reaction is not observed. In cases where enough of the changed nucleolar matter has been present, we get a slight reaction of the latter; in this case we may deal simply with the oxidation of the decolorized fuchsin. Stronger doses are apparently necessary to destroy completely the chromatin reaction of the larger nuclei.

Tests for Lignin, Suberin and Cutin

The persisting reaction given by lignin, suberin and cutin gave the idea of a possible test for these. Results quicker than those when using the long paraffin procedure seemed desirable. Free-hand sections proved satisfactory for a rapid diagnosis.

Method: Cut sections and transfer to distilled water. Do not hydrolyze if a reaction is desired for lignin, suberin and cutin only. If, on the other hand, a chromatin reaction is also wanted, hydrolyze as usual. Stain one hour, more or less as the case requires, in the fuchsin sulfurous acid. Examine in the sulfurous bath. Pass thru a few changes of the latter, rinse in distilled water and preserve in glycerin for longer mounts. For permanent mounts rinse in distilled water, counterstain with orange G or some other counterstain, dehydrate, clear and mount in gum Dammar. Permanent mounts of

free-hand unfixed sections, altho perfectly possible, are not recommended, because of lack of proper fixation. They are not worth while.

Satisfactory results were thus obtained with roots of *Pandanus*, *Amaryllis* and *Cineraria*, with leaves of *Tradescantia*, *Geranium*, and *Cineraria*, and with the petioles of the two latter ones. The reaction is given by the xylem—both tracheae and tracheids (i. e. lignin); by the walls of the endodermis and sclerenchyma cells (i. e. suberin); the cutin layer and here and there hairs (i. e. cutin). The suberin reaction is very striking in *Pandanus* where the heavily stained bunches of sclerenchyma cells stand out in a colorless field of parenchyma. The outer walls of guard cells, those that actually bind the opening (generally considered cellulose) also react for some reason.

Since the appearance of the purple color in Feulgen's reaction is based on Schiff's reaction for aldehydes, let us now try to explain the reason and the possibilities of its presence in lignin, suberin and cutin.

Mehta's (1925) extensive chemical studies of lignin clarify some of our results. In discussing the usual phloroglucin test for lignin, she says, p. 959: "There seems to be little doubt that the active grouping concerned in the coloration is of the nature of an aldehyde and not a quinone"; and further: "Phloroglucin is thus not a stain for the major part of the aromatic constituent which is the precursor of the one present in the lignocellulose, but rather for an aldehyde of the nature of hadromal. This aldehyde is always associated with lignin".

The exact chemistry of cork is unknown. A considerable part of it is composed of fatty acids and little ash is left. Aromatic substances are usually present. Gilson (1890) finds that in *Quercus* all the layers of cork cells give the red reaction with phloroglucin. Czapek (1922) finds that the middle lamella gives the reaction, but mentions that hadromal has not yet been detected in cork. A few pages further, however, there seems to appear discrimination: "Das Hadromal, der aromatische Aldehyde des Holzes, scheint wie erwähnt, ein regelmässiger Bestandteil der Korkmembranen zu sein". Kügler (1884) found that the middle lamella is usually lignified between the layers of suberin, which may be surrounded by cellulose; and the latter is frequently more or less lignified. He also found small amounts of vanillin and coniferin in cork (1913). Vanillin was later detected by Bräutingam (1898) and Thoms (1898).

Considering Mehta's findings concerning the active agent in the phloroglucin test for lignin, Gilson's microchemical observations,

those of the three other investigators, and Czapek's second sentence about the presence of hadromal in suberin, we may well suppose that the first is a misprint. Nevertheless, even if we consider the question of hadromal unsettled for cork, the presence of other aldehydes justifies our reaction. Vanillin is an aldehyde and the glucoside, coniferin, is easily oxidized to vanillin glucoside which yields vanillin when hydrolyzed.

Cutin is a waxy substance, which some chemists consider to be analogous with suberin, while others point out few differences. Géneau de Lamarlière (1903), (the photostat of whose article was obtained after the completion of these tests) was apparently the first to discuss the aldehyde reaction of cutin with Schiff's reagent. He also observed this reaction with lignin and suberin, but has made no application of it. This author excludes the possibility of hadromal in cutin. Further studies of these substances offer an explanation for this reaction. Mehta says (p. 996) "Lignification sets in very early in the life history of plants and occurs in ground tissue, vascular bundles, medulla and cuticularized cells".

These three substances give the same reaction, not necessarily because they are identical, but probably because the reaction is based on an aldehyde or aldehydes which are a constituent of the molecules of all of them. It has to be emphasized that, as in other cases, microchemical tests are of great value only when correlated with either anatomical structures or physiological appearances.

The Chromatin of Plants and Unicellular Organisms

Previous studies. Feulgen's reaction has been tested out on few protozoa. Besides his own findings (Feulgen and Rossenbeck, 1924), positive results have been obtained in *Valkampfia*, *Amoeba*, and *Actinospherium* (Bogdanovicz, 1930), in *Paramecium caudatum* and *Dileptus anser* (Margolena, 1931), while Polyansky and Petruschevsky (1929) found that certain granules of the central body of Cyanophyceae contain chromatin.

Feulgen, who developed this technic, originally questioned the possibility of plant tissue reacting because of the general opinion that vegetable organisms contain "Hefenucleinsäure" instead of the thymonucleic acid. He mentions that alleged nucleic acids have been extracted from plants only in two cases, namely from yeasts, and from wheat embryos. In the second instance, however, he points out that the acids extracted were apparently derived from the cytoplasm, not the nucleus, for nucleic acids are not easily extracted, and could hardly be dissolved by the water surrounding the crushed

cells. The acid isolated from wheat Osborne and Harris's (1899), "Triticonucleinsäure,"[1] differs from the thymonucleic acid, and is considered of the same type as that of yeasts. This type, therefore, was thought at first to be representative of the vegetable kingdom.

In estimating nuclear reactions and discussing their acids one has to remember that Feulgen stressed very strongly the fact that only three kinds of cells thus far permitted critical analysis, that is permitted the isolation of nuclear matter from the cytoplasm—namely, white blood cells, avian erythrocytes and spermatozoa. He adds that "Hefenucleinsäure" has been obtained from yeasts, altho their nuclei probably were not free from the cytoplasm.

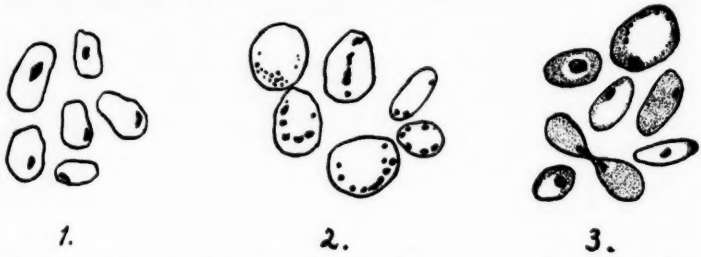

Fig. 1.—*Saccharomyces monacencis*, 30 hour culture, showing Feulgen's reaction.
Fig. 2.—Same, but not hydrolyzed.
Fig. 3.—Same, stained with Heidenhain's hematoxylin.

Nucleic acids are composed of phosphoric acids, protein bases, and carbohydrates. Of these, Feulgen distinguishes two kinds: thymonucleic acid, present in animals, which contains a hexose radical; and "Hefenucleic" acid, containing a pentose radical, and which has been isolated from yeasts. Feulgen (1924), obtained negative results with bacteria and yeast nuclei, and he tested sections of wheat embryos, fully expecting the same findings; but unexpectedly obtained a very clear and definite reaction. Westbrook (1930), moreover, obtained a positive reaction with *Allium* (onion), *Asplenium* (spleenwort) and a red alga, *Rhodymesia palmata;* but he mentions that neither yeasts nor bacteria give the reaction.

The present findings. Various bacteria have been studied here by this method for several months. An apparent purple reaction has been observed, but the writer has not yet come to a definite conclusion as to its meaning. For the reasons discussed above the writer's observations of yeasts are of special interest.

Three strains of *Saccharomyces ellipsoideus*, three of *S. monacencis*

[1]See Feulgen and Rossenbech, 1924.

and one of the type of *S. cerivisiae*, 1, 2, or 6 days, or a month after incubation, fixed only by heat, gave invariably a positive reaction. Altho yeast cells suffer from hydrolysis, a fusiform body staining paler than the chromatin observed in higher organisms and protozoa is usually present, see Figs. 1–3.

Unhydrolyzed smears of these 7 strains were also stained (for controls) with the fuchsin sulfurous acid and gave results very different from those described above, see Fig. 2. These yeasts which have not suffered shrinkage from hydrolysis, are larger and show their typical shape better than the others. No signs of the purple body can be distinguished. Instead more or less numerous reddish, (not purplish) stained granules lie in the cytoplasm, frequently distributed as if they were arranged toward the periphery of the vacuole. These are probably the metachromatic granules and the restoration of the red color is an oxydation phenomenon. The vacuole itself, which is clearly outlined when Heidenhain's hematoxylin is used, does not show well here; see Fig. 3.

The "nuclear vacuole" of Wager and Penistion (1910), filled with chromatin grains and a homogeneous nucleolus outside of the vacuole, but connected with it, is hardly possible considering our reaction. The results of my tests conform with Guillermond's (1920), cytologic observations on yeasts. A more complete paper on the chromatin of yeasts will appear elsewhere. At present it suffices to say that either the yeast cells contain some of the same kind of chromatin as do nuclei of other organisms, or else Feulgen's reaction is not so specific for one particular kind of nucleic acid as claimed by its author. For the present we must regard Feulgen's reaction as of value for cytological staining rather than as an *absolute* microchemical reagent.

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A NEW METHOD OF DIFFERENTIATING GENTIAN VIOLET WHEN USED AS A SOMATIC CHROMOSOME STAIN

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ABSTRACT.—**The addition of 0.5% of picric acid crystals to the dehydrating alcohols makes possible a much better differentiation of gentian violet than has previously been obtainable. The final differentiation occurs in clove oil. The method was especially designed for use on the root tips of plants normally growing in arid regions.**

The root tips of plants which inhabit desert or very arid regions, or of plants such as *Godetia* and *Hemizonia*, which begin growth late in the season and commence flowering after the ground has become quite dry, are difficult subjects for the cytologist. The root tip cells of such plants are congested with foodstuffs and other secretory products and it seems that the higher percentage of dissolved salts entering the roots tends to lower the capacity of the chromatin of both resting nuclei and chromosomes to receive and especially to retain the usual chromatin stains. When using iron hematoxylin following a procedure which would yield satisfactory results with, say, *Allium* or other liliaceous plant, one at once discovers that the cytoplasm tends to overstain and the chromosomes lose the stain too easily and quickly. A killing and fixing fluid which overcomes this difficulty is not known to the writer, while many solutions aggravate matters by enhancing the staining capacity of the cytoplasm and its inclusions. When Heidenhain's iron hematoxylin was used on root tips of various species of *Hemizonia*, it was discovered that in some species one pair, or sometimes two pairs, of homologous chromosomes often were completely destained while the other pairs remained dark enough to be recognized in the deeply stained cytoplasm. As such results would naturally lead to the making of erroneous chromosome counts, it became necessary to devise a new staining method for the type of material described above, since none of the methods in common use gave results which were dependable.

For the killing and fixing of the root tips, the following fluids afforded excellent results: a medium chrom-acetic to which is added 2% of maltose or urea; Champy's fluid; or Navashin's fluid. N-butyl alcohol should be substituted for xylol preceding infiltration in paraffin as xylol tends to overharden the tissues. Transverse sections (cut at 11μ in Hemizonia and related genera) are preferable.

The new method, as finally revised, is as follows:

1. Bring slides down to water.

2. Stain for about 15 minutes in a freshly prepared 1% aqu. dist. solution of gentian violet. In our trials, we used a stain labelled gentian violet, manufactured by the National Aniline and Chemical Co., Commission certification No. NC-6, the total dye content being given as 81%. It is essential, for the best results, that a freshly prepared solution be employed. The optimum time in the stain needs to be determined for each species. With some, 10 minutes suffices, while others may require as long as 30 minutes.

3. Transfer to water and wash off excess stain. Henceforth the slides must be handled individually. No harm is caused by leaving the slides in the water for as long as 20 minutes.

4. Dehydrate in a series of alcohols, 10–30–70–83–95%, to each of which is added 0.5% of picric acid crystals. About 25 seconds in each fluid is sufficient, as there is little or no danger of extracting too much stain. The slides must be kept moving back and forth in the vessels containing the acid alcohols. The excess stain will be removed in the 70% solution.

5. Transfer to 95% ethyl alcohol, to which is added about 3 drops of ammonia per 100 cc.

6. Complete dehydration in absolute alcohol.

7. Differentiate in pure clove oil. This step is the critical one in the schedule. With some species only a few seconds are permissible, while with others a longer time is required. Two or three trial slides will quickly reveal the optimum time. If action is too rapid, leave in the stain a longer time. It is important to keep the slide in motion while in the oil in order that differentiation may proceed equally in all sections, especially if there is a large number on the slide.

8. Wash thoroly in xylol to remove every trace of oil.

9. Xylol, then mount in balsam or other preferred medium.

The results secured by this method are, in the writer's experience, much superior to those afforded by Newton's gentian-violet-iodine method. By the latter method, the somatic chromosomes usually have a disagreeable transparency and for this reason it is occasionally difficult to judge whether one is viewing a single chromosome or two overlapping elements. The newer schedule gives a certain degree of transparency, but the chromosomes are more sharply delimited and the making of counts is greatly simplified, especially in species where variations in number and morphology occur. The cytoplasm is faintly stained in some species, more so in others; in any event, a counterstain is hardly desirable.

Lignified structures are also sharply stained by the method; crystal

violet gives a slightly darker stain to such elements than does gentian violet.

Upon being informed by the Chairman of the Committee on Standardization of Biological Stains that "all the gentian violet sold by the National Aniline and Chemical Company is actually crystal violet," we were led to repeat our earlier but rather unsatisfactory attempts to use other crystal violets in place of the one labelled gentian violet. Two facts having an important bearing on the successful use of crystal violet by the method outlined above came to light: first, the picric acid alcohols must not contain traces of any other stains (traces of safranin were found to extract crystal violet from chromatin, except in prophase stages), and second, the 1% aqueous crystal violet solution should be allowed to stand at least three weeks before being used. We employed a crystal violet sent out under that name by the same firm from which the "gentian violet" was procured (Commission certification NC-2; total dye content, 84%). The chromosomes were darkly stained, but everywhere there was a disagreeable reddish transparency which was particularly evident in the nucleoli. Personally, we would much prefer to use NC-6. Evidently the two batches are not identical.

We also experimented with a methyl green made by the same company (Commission certification NG-3; C. I. 684) and secured excellent results. The dry dye had been in stock for some time, hence had doubtless undergone a slight change in stability as the chromosomes were a dark purple color, with tinges of green in prophase nuclei. Methyl green worked better on certain species of *Lagophylla* upon which the gentian violet (NC-6) was not wholly satisfactory. The cytoplasm retains the stain so slightly that sections of root tips, after being taken from clove oil after an immersion of about 15 seconds and washed in xylol, are almost colorless to the eye.

Later Dr. Conn forwarded two samples of stains. One was from the Coleman and Bell Co., (certif. No. CB-8), labelled gentian violet, but declared to be "about a methyl violet 2B". It gave most satisfactory results. The other was a methyl violet 2B from the National Aniline and Chemical Co. (certif. No. CMy-1). It gave the same disappointing results as does crystal violet, NC-2. The conclusion obviously is that no dependence can be placed on labels; the suitability of the stain for this procedure can be judged only by actual trial, care being taken that the tissues were fixed in a fluid containing chromic acid.

The method outlined above has been used with the greatest success with over sixty species and forms of *Hemizonia*, and with many

species of *Madia*, *Lagophylla*, *Fouquieria*, *Idria*, *Godetia* and *Sedum*. With these forms no other staining method has proved fully satisfactory. The keeping quality of the stain is not known; slides stained seven months before this paper was written have retained their original sharpness, and some had been intentionally exposed to sunlight.

The method was worked out while the writer was engaged in a cytological study of the genera belonging to the tribe Madinae of the family Compositae for Dr. H. M. Hall, of the Division of Plant Biology, Carnegie Institution of Washington.

DIFFERENTIAL STAIN FOR ACID FAST BACTERIA AND SPORES

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ABSTRACT.—**A modified method of staining acid-fast organisms is described. After staining with carbol-fuchsin as usual in the Ziehl-Neelson method, wash with water and while the slide is still wet cover with a saturated acetone solution of malachite green for three to five minutes. Wash and examine. The acid-fast organisms and spores are red in a green background. If the smear is thick and appears too dense, dry for three minutes and hold over the mouth of a bottle of ammonia until decolorized to suit. Upon exposure to the air the green returns. This can be prevented by keeping the smear alkaline, by the addition of sodium bicarbonate.**

A second method is described for use with sputum in which acid-fast organisms are scarce. It permits the examination of thick smears and therefore increases the chances of finding tubercle organisms when few in number. Stain with carbol fuchsin as in the Ziehl-Neelson method. Decolorize with 30% phenol-disulfonic acid in water for a few seconds or until decolorized. Wash and examine at once. If color returns upon washing decolorize again. The tubercle organisms appear red in a colorless background.

The method to be described depends upon the action of certain dyes such as malachite green or brilliant green on basic fuchsin. The addition of such dyes to basic fuchsin results in the masking of the color of the latter dye. This eliminates the necessity of a separate decolorizer for the removal of the fuchsin from the non-acid-fast material and cells. A second innovation is the use of ammonia fumes to reduce the intensity of the malachite green in thick smears.

PROCEDURE

(1) Stain either hot or cold with basic fuchsin by the Ziehl-Neelsen technic. This stain is usually prepared with phenol, constituting the ordinary carbol-fuchsin, but it has been our experience that the dye prepared without the phenol stains well and the slides have less precipitate, are cleaner and from this standpoint preferable. (Certified basic fuchsin from the National Aniline and Chemical Co., has been used in our work.)

Upon removal from the carbol-fuchsin immediately wash in water, being careful not to permit the smear to dry.

(2) Cover wet smear with a saturated acetone solution of malachite green for three to five minutes depending on the thickness of the smear. Add more of the malachite green solution as evaporation occurs. (The stain used by the writers was not certified but obtained from the above named company, marked "Shultz No. 495, Lot No. 1128.")

Evaporation is rapid and this is an objection to the method. This objection may be overcome by placing the slide in a covered jar of the dye. Such dye cannot be used indefinitely, but must be replaced when results appear unsatisfactory. Evaporation on the slides is reduced if the malachite green acetone solution contains 10 to 20% distilled water. An aqueous solution of malachite green is not as satisfactory as an acetone solution.

(3) Wash in water and examine. The acid-fast organisms and spores are red in a green background.

(4) If the smear is too heavily stained, allow to dry three minutes and decolorize half of smear to the density desired by holding over the mouth of a bottle of ammonia.

Results

Complete decolorization of the malachite green may be brought about and in some cases this may be desirable but it should be remembered that such decolorization may result in the fuchsin becoming visible again in which case other than the acid-fast organism may appear red. This happens occasionally but not always and indicates that the fuchsin is not washed away but is masked by the malachite green. In some cases the fuchsin does not become evident upon the decolorization of the malachite green. The fuchsin is less likely to appear if, in step 4, the smear is dried three minutes or more. If dried more than three minutes the slide may be practically decolorized without the fuchsin appearing. Apparently the basic fuchsin has been permanently changed.

Upon standing, after treating with ammonia, the smear becomes green again and the acid-fast organisms appear red in a green background as originally. The return of the green can be checked by the addition of 5% sodium bicarbonate. The malachite green stained material is transparent and the acid-fast organisms stand out in it almost as clearly as in the smear decolorized by ammonia fumes. In sputum in which no acid-fast organisms are found it may be ad-

visable to remove the immersion oil with xylol, to decolorize thoroly with ammonia and reexamine. Oil covered parts of the smear resist decolorization.

Non-acid-fast organisms are readily penetrated by the malachite green and its effect in masking the fuchsin present is the same as its action outside the cell.

This method has been used by a number of workers, both experimentally and in the routine examination of sputum. The method has been in use in the San Joaquin General Hospital, California, in comparison with the Ziehl-Neelsen technic. Those carrying on the work there report (personal communication) that, "We both feel that we can only say that in our experience it is as satisfactory as the Ziehl-Neelsen and we find it less trouble. It is certainly easy to read, the red bacilli seem to stand out especially well." In routine diagnosis they ran 172 pairs of slides, one of each pair stained by one of the two methods and "the findings in each case checked exactly."

Experimental results support these conclusions. In comparing the Ziehl-Neelsen method with that described, experiments were carried out to determine both the average number of organisms visible per field in smears containing many tubercle bacilli and also the average time required to count a definite number of bacilli in smears containing few bacilli.

A series of twenty uniform smears were made from one sample of sputum. The sputum was stirred and a measured amount spread evenly over one square centimeter of the slide. After fixing, the smears were stained three to five minutes in streaming carbol-fuchsin. Ten of the smears were stained by the Ziehl-Neelsen method and ten by the method described. The number of tubercle organisms appearing in twenty microscopic fields on each slide were counted. The average number of organisms per field for the two hundred fields examined on the smears stained by the Ziehl-Neelsen method was 11.9, for the smears stained by the new method 10.7.

A second experiment was made to compare the time required to find a definite number of organisms in smears stained by the two methods.

A sputum containing few tubercle organisms was obtained. Twenty uniform smears were prepared as in the previous experiment. Ten were stained by the Ziehl-Neelsen method and ten by the method described. The microscope was focused on each slide and then the time necessary to count ten acid-fast organisms recorded. The average time required to count ten organisms on the slides stained by the

Ziehl-Neelsen method was two minutes twenty-six seconds, on the slides stained by the new method one minute forty-two seconds.

A Procedure for Thick Smears

As with all acid-fast staining of sputum, the thick and thin areas require different treatment to bring about satisfactory staining. An attempt to find a satisfactory method of staining thick lavers of sputum was made. More organisms will be present on the slide in a thick smear and the accuracy of diagnosis increased. In the staining of thick smears the following method proved useful:

Stain with carbol-fuchsin as usual. Wash. Decolorize with 30% phenol-disulfonic acid in water for a few seconds or until decolorized. Wash and examine at once. If color returns upon washing, decolorize again.

Upon addition of the acid, the fuchsin becomes yellowish and finally colorless. The tubercle organisms stand out in a colorless background. If the acid is left on too long the acid-fast organisms become decolorized.

In comparing this method with the Ziehl-Neelsen and the malachite green method the following results were obtained: The average numbers of acid-fast bacilli counted in two hundred microscopic fields were 14.4 for the phenol-disulfonic acid method, 11.9 for the Ziehl-Neelsen method and 10.7 for the malachite green method. The average time required to count ten organisms on each of ten slides was forty-seven seconds for the phenol-disulfonic acid method, two minutes twenty-six seconds for the Ziehl-Neelsen method and one minute forty-two seconds for the malachite green method. The slides compared were made as uniform as possible.

The phenol-disulfonic acid method should prove superior in the staining of thick smears. If smears are made too thick they wash off from the slide. The phenol-disulfonic acid method does not give any indication of the other organisms or types of fibres present. For this reason it is not satisfactory as a routine stain. A counterstain may be applied after a microscopic examination and this data obtained.

The authors are indebted to Miss Alma Strobel and the laboratory staff of the San Joaquin General Hospital for comparing the new method with the Ziehl-Neelsen method in routine diagnosis.

NOTES ON TECHNIC

CONCERNING DEHYDRATING AGENTS FOR PARAFFIN EMBEDDING.—O. C. Bradbury[1] finds that in replacing ethyl alcohol with iso-propyl in the dehydration of animal tissues, the hardening effect of the former is omitted. Besides his own favorable experience he observed that a class of new students succeeded in preparing better slides when iso-propyl alcohol was used instead of the usual method. The alcohol in question is obtained 98 to 99% pure, and this may be used as absolute or, if desired, it may be distilled with rock lime.

Dr. C. Zirkle[2] recommends normal butyl alcohol for the infiltration of woody plant material into paraffin. The decided advantage of this method is the elimination of *both* hardening agents, the higher percentage alcohols, (ethyl alcohol over 50%, is not used in this procedure) and xylol. This is possible because a mixture of ethyl alcohol and butyl alcohol is miscible with water, while butyl alcohol alone is not, and because normal butyl alcohol is soluble in paraffin.

The method seemed worth broader application and has been tested on animal tissue in this laboratory. A guinea pig's kidney fixed in Zenker's fluid, run up without xylol, and with normal butyl alcohol, yielded very satisfactory and well stained sections. The iso-butyl alcohol technic was not tried.

The technic employed is very satisfactory and seems well worth recommending. Unless iso-propyl alcohol has some special advantages over normal butyl, the latter undoubtedly should be the more generally used.—LUBOV MARGOLENA, Geneva, N. Y.

ERYTHROSIN FOR STOUGHTON'S THIONIN ORANGE G.—R. H. Stoughton's[3] thionin-orange-G technic, is a method for the differential staining of fungal parasites in plant tissues. The organisms appear purple against the yellowish green walls of the host, and the bluish lignified elements. The writer has modified this technic by using erythrosin as counterstain in addition to the orange G, in order to obtain more differentiation of the host. Light green has been found more suitable for differential purposes in this procedure than the

[1]Bradbury, O. C. A new dehydrating agent for histological technique. Science, **74**, 225, 1931.

[2]Zirkle, C. The use of N-butyl alcohol in dehydrating woody tissue for paraffin embedding. Science, **71**, 103–104, 1930.

[3]Stoughton, R. H. Thionin and orange G for the differential staining of bacteria and fungi in plant tissues. Ann. Appl. Biol., **17**, 162–164. 1930.

orange G. *Plasmodiophora brassica* in cabbage roots, fixed in sublimate acetic, *Peronospora* on pigweed, and *Puccinia* on *Aster*, fixed in Allen's a-3, served for material.

The modified staining method is as follows: paraffin sections are run down to water and stained in Stoughton's thionin[4] (thionin, 0.1 g.; 5% aqueous phenol, 100 cc.), for one hour. Then rinse in water, counterstain in 0.5% light green in 95% alcohol until the section appears green; wash in water, which will take all the green color out of the sections, except in the xylem, pass thru 95% to 100% alcohol, and counterstain in a mixture of orange G with erythrosin. Use about 1 part of saturated orange G in 100% alcohol to 2 parts of saturated erythrosin in clove oil. Clear in xylol and mount. (Erythrosin NEr-2 has been used.)

Results: Yellow cutin and spore walls, yellowish pink cellulose, pink cytoplasm, green lignin and reddish middle lamella against the purple hyphae and spores.—LUBOV MARGOLENA, Geneva, N. Y.

ON THE RIPENING OF HEMATOXYLIN FOR HEIDENHAIN'S METHOD.—Text books on microtechnic ordinarily mention the necessity of ripening hematoxylin, and most workers, therefore, take the process as inevitable and proceed accordingly. Chamberlain,[5] however, says that American hematoxylin ripens faster and "may reach its maximum efficiency in 2 to 3 weeks."

Some time ago, in testing certain batches of hematoxylin, the writer happened to notice that the lot bearing the certification number FH-5 was ready for use within a few hours after its preparation. More recently the same fact seemed to be true for the lot certified as FH-8. Since the original description and application of hematoxylin staining came from Europe, where the product on the market has been considered poorer (that is, unoxydized) a sample of Grübler's hematoxylin and a number of certified American samples, were tested as to the time required for "ripening." The crystals of the Grübler product, lighter than the American, dissolved somewhat slower, and seemed to substantiate the idea that European hematoxylin might require weeks or months to ripen, as stated in books. A sample of hematein has also been used for comparison.

The depth of color of the crystals and of 4-hour solutions is given here in a descending order: hematein FHi-2, hematoxylin FH-5, FH-9, FH-8, FH-6, and Grübler's hematoxylin. The same order of

[4]National Aniline thionin (Cert. No. NT4) has been used.

[5]Chamberlain, C. J. Methods in Plant Histology. Univ. of Chicago Press. 4th ed. p. 44.

color intensity showed after 20 days, except that FH-5 was then slightly darker than FHi-2.

The hematoxylins and hematein were tested in 0.5% aqueous solutions, all prepared simultaneously, at the same room temperature, and on the same material (root tips, Flemming's fixation; cyclamen buds, Allen's fixation; and kidney, Bouin's fixation). The staining solutions were 4 hours old, 29 hours and 20 days old, respectively. The sections were mordanted 30 minutes, and stained an hour and a half, and all gave entirely satisfactory results.

These tests prove that American hematoxylins do not require long periods of ripening, a few hours being entirely sufficient; or more precisely one may say that a good hematoxylin is ripe as soon as dissolved. A similar observation has been made in regard to one European sample, but as no other foreign hematoxylin has been tested, no general conclusion can yet be drawn concerning the hematoxylin on the market in Europe.

These observations suggest that hematein, altho considerably more expensive than hematoxylin, has no real advantage over the latter for the microscopist.—Lubov Margolena, Geneva, N. Y.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the October number of this journal.

Stains Certified September 1, 1931 to November 30, 1931*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Crystal violet...	LC 4	92%	As histological and bacteriological stain	Sept. 1, 1931
Thionin.....	NT 4	92%	As histological stain and for frozen tissue.	Sept. 1, 1931
Eosin Y.....	LE 7	84%	As histological counterstain	Sept. 8, 1931
Eosin Y.....	NE 8	92%	As histological stain and as constituent of blood stains	Sept. 8, 1931
Malachite green.	NMg 3	75%	As histological stain	Sept. 18, 1931
Eosin B.....	NEb 5	80%	As histological stain	Sept. 28, 1931
Cresyl violet....	NW 4	96%	As histological stain	Sept. 30, 1931
Phloxine.....	NPh 1	87%	As histological counterstain	Oct. 15, 1931
Indigo Carmin..	NI 4	91%	As histological stain	Nov. 10, 1931

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order direct from STAIN TECHNOLOGY, at a price of 6 cents each.

CARPENTER, D. C., NEBEL, B. R. Ruthenium tetroxide as a fixative in cytology. Science, **74**, 154–155. 1931.

Ruthenium tetroxide is proposed as a fixing agent, superior, in certain respects to osmium tetroxide. It has been used for pollen mother cells of *Tradescantia zebrina* Hort and found to be extremely useful in demonstrating the chromonematic structure of chromosomes. Proceed as follows: smear anthers between two slides, and immediately apply diluted fixative for 3 minutes. Pour off and replace with a drop of Linder's medium: glycerin 40; lactic acid 20; phenol 20; H_2O 20%; cover and seal. The chromonemata should appear dark on a gray cytoplasm. Carmin may be added to Linder's medium, or the slides may be stained otherwise.

Ruthenium tetroxide has the disadvantage of decomposing very readily. It fixes the outer cell layer very successfully, but appears to decompose before reaching the inner layers of tissue. For least decomposition, use chlorine water as solvent. Prepare stock solution by breaking a 1 g. ampul into 100 cc of saturated chlorine water. For use dilute 1:20 with distilled water, or 0.25 to 1% of formic, or acetic acid solution for the fixative.

COOK, J. E., MEYER, H. M. and TUREEN, L. L. Reticulocyte count preparations: An improved technic. J. Lab. & Clin. Med., **16**, 1224. 1931.

By means of this method smears for the reticulated blood cells may be easily made at the bedside. It does not require any special apparatus and the slides are quite free from precipitate. Prepare: A) a 1% solution of brilliant cresyl blue, (use a little heat), in physiologic salt solution, cool and filter; and B) a 1% solution of potassium oxalate in physiologic salt solution. Add one drop of B to five drops of A in a small container, just before using. Draw a small drop of blood into a capillary pipette of the kind used in the Sahli Hemometer. Draw an equal amount of the oxalated stain and mix thoroly by alternately blowing the fluid out onto a glass slide (slide as used for hanging drop preparation is very suitable for the purpose), and redraw into the pipette several times, avoiding air bubbles. Make a thin smear of this mixture on a slide and dry quickly. Counterstain with a light Wright's stain if desired.

Wright's stain and a ruled ocular or one fitted with a small diaphragm facilitate the count. A routine and reticulated count can thus be made simultaneously.

COOK, W. R. Ivimey. A simple method of staining fungi in fine roots. Watson's Micr. Rec., **19**, 1930.

Roots are fixed in Bouin's fluid and, after washing treated *in toto* with iron-alum and stained for 1 hour in Heidenhain's hematoxylin, differentiated in acid alcohol, crushed on the slide, and passed thru the grades of alcohol into xylol.—From Biol. Abstr. **5**, No. 16642, 1931.

HOLLANDE, A. C. L'insolubilisation des urates figurés dans les coupes histologiques. Bull. d'Histol. Appl., **8**, 176–178. 1931.

This is a modification of Courmont-André's method for the demonstration of urates by means of ammoniacal silver nitrate, which blackens uric acid and urates. It has the advantages of better penetration and it does not leave any precipitate, which may naturally lead to errors in interpretation.

Proceed as follows: fix in equal parts of 1% silver nitrate and 4.4% formalin, (the latter may be neutralized with calcium carbonate), for 12 to 24 hours, in darkness. Wash in distilled water for 24 hours, changing it several times. Dehydrate in 75, 90, 95 and 100% alcohol and embed.

Sections may be stained, if desired, in hemalum 10 minutes; wash several hours under tap water; apply 1% aqueous orange G or eosin, as preferred, for 30 minutes to 1 hour; rinse in distilled water. Transfer to 0.5% phosphomolybdic acid, rinse in water, and stain in 0.12% aqueous light green, for 1 to 10 minutes. Differentiate rapidly in 96% alcohol. Dehydrate with iso-amyl-alcohol, pass thru xylol and mount in balsam.

Excellent results were thus obtained with adipose tissue surrounding the urate-containing cells and bacteriocytes of *Periplaneta orientalis* L. Urates appear black, chromatin blue, protoplasmic inclusions red or orange, depending on the counterstain used, collagen and products of secretion light green.

HUDDLESON, I. F. Differentiation of the species of the Genus Brucella. Am. J. Pub. Health, **21**, 491–498. 1931.

Three groups or species of *Brucella* (bacilli of contagious abortion, malta fever etc.) may be distinguished according to the growth inhibiting action of thionin in a final dilution of 1:30000 and pyronin 1:20000, in beef liver infusion agar at pH 6.6. The stain powder and the distilled water used in preparing the solutions are sterilized before coming in contact. The writer states that dyes from certain manufacturers are unsuitable, but does not give the source from which those employed were obtained.

Br. militensis (Bruce) grows on both the thionin and pyronin media, while *Br. abortus* only on the one containing pyronin, and *Br. suis* only in the thionin medium. Methyl violet or basic fuchsin, in suitable dilutions, completely inhibit the growth of *Br. suis* for 72 hours, but only slightly that of *Br. militensis* or *Br. abortus*.

The capacity to reduce basic fuchsin is a typical character in distinguishing between 2 classes of *Br. abortus*. One completely reduces basic fuchsin (1:25000, in beef liver infusion agar), while the other reduces it but slightly. The latter is apparently pathogenic to man.

When more than one species of *Brucella* occurs in the infected material, the dye method and H_2S metabolism determination will separate and identify each species.

KISSER, J. Eine neue Farbstoffkombination, das Kallichrom nach Dr. Gross. Mikroskopie f. Naturfreunde, **9**, 95, 1931.

Dr. Gross' "Kallichrom" is a combination of cresyl violet and auramin. It may be purchased from Dr. Hollborn (Dr. G. Grübler) Leipzig S 3, Hardenbergstr. 3, Germany. It is prepared by mixing saturated solutions of both stains until a cherry red color, without a yellowish or purplish tinge, is obtained. Kallichrom is a differential histologic stain for plant tissue; nuclei are dark blue, woody elements green or blue, and cellulose red or pink.

Dr. Kisser describes the process as follows: transfer unfixed sections from H_2O to the above mentioned solution diluted 1 to 3 or more, for few seconds or longer as the case may require. Rinse in H_2O, pass thru 70% alcohol, differentiate in 90%. Treat with turpentine oil and xylol and mount in balsam. Eau de Javelle is recommended for clearer results prior to the distilled water and before the staining; this naturally destroys the cell content, but the material is not supposed to be of cytologic value. Tests with fixed plant material have not yet been completed.

Dr. Gross finds that the stain is also a differential one with animal material. Nuclei appear blue, cytoplasm pink, connective tissue blue, bone light blue and blood cells green.

KOHL, E. J. and JAMES, C. M. A method for ripening hematoxylin solutions rapidly. Science, **74**, 247. 1931.

Quartz mercury vapor light is recommended for the fast ripening of Ehrlich's Delafield's, and Heidenhain's hematoxylins. The solutions are exposed in a very wide, somewhat shallow, evaporating dish to a powerful quartz mercury vapor light, and stirred frequently.

Ehrlich's and Delafield's hematoxylins should be at a distance of about 2 feet from the light and will ripen in 2 and 3 to 4 hours. Shorten the exposure in the case of Heidenhain's fluid.

Very vigorous hematoxylins are thus obtained.

KOEHRING, VERA. Thermal relations in the neutral red reaction. J. Morph. & Phys., **52**, 165–194. 1931.

Neutral red staining, as observed in plant and animal material, closely approximates in its reactions to temperature the reactions of enzyme to the same factor. These re-

actions were studied in dilute solutions of neutral red in regard to the following: live cells of *Daphnia pulex*, legume seeds grown to seedlings with the aleurone layer showing the color and all stages of protein hydrolysis; starfish eggs, treated vitally and supravitally with neutral red in relation to their artificial parthenogenesis induced by momentary changes in temperature; and in Palaeomonetes, where stomach, carapace, and gills show the color macroscopically. Neutral red is worth testing as an enzyme indicator. Its possibilities as a tool for investigation of enzyme activity in vivo, seem very promising.

PETRAGNINI, G. Metodi rapidi di colorazione dei corpi del Negri. Boll. Inst. Sièrot Milanese, **7**, 557–561. 1928.

This is a rapid method for the coloration of Negri bodies. For mordanting prepare: A) dissolve 3 g. of ground potassium alum, 0.5 g. of lead acetate, 3 drops of acetic acid in 100 g. of H_2O; and mix with C) 7 g. of tannic acid, 2 g. of ferric chloride, 35 g. of methyl alcohol, 15 cc. of H_2O. Filter after 2 to 3 days and dilute with 20 to 50 or more volumes of methyl alcohol.

Method A. Pass paraffin sections thru xylol and absolute alcohol. Treat 5 to 10 seconds with the dilute mordant, wash rapidly in absolute and in 95% alcohol; stain 10 to 20 seconds in 0.5% eosin in 50% alcohol; and wash slowly in H_2O; stain 1 minute in Mayer's hematoxylin, wash rapidly; stain with methylene blue until violet, dry with filter paper, agitate in 0.25% N/2 NaOH for 15 to 20 seconds; wash with 90 to 95% alcohol until a general blue, absolute, xylol and neutral balsam.

Results: nerve cells blue, nuclei, capillaries and endothelia brilliant red, nucleoli dark blue, Negri bodies eosin red.

Method B. Treat with mordant diluted 100 to 200 volumes of water, wash with absolute, then 75% alcohol; stain with eosin as above, or acid fuchsin, wash; then 95% alcohol, absolute, xylol and mount.

Results: Cell bodies pink, Negri bodies red.

Method C. Treat with mordant 1 volume in 20 to 40 volumes of methyl alcohol for 5 to 10 seconds, then acid fuchsin, 1 minute, wash; stain with 0.2% aqueous indigo carmin for 5 to 20 seconds, wash; then 95% alcohol, absolute, xylol and balsam.

Results: The chlamydozoic structure of Negri bodies is well revealed by this method for the carmin partially displaces the acid fuchsin.

ROGERS, M. W. New silver methods for paraffin sections. Anat. Rec., **49**, 81–85. 1931.

Serial sections showing clear differentiation between nerve fibers and connective tissue may be obtained as follows: Fix for 7 days or longer in 10 to 20% formalin or Bouin's solution. Wash formalin material in running water for at least 1 day, pass the Bouin material thru several changes of 70% alcohol, until the yellow color disappears. Dehydrate at least 24 hours in 80% and then in 90% alcohol, each containing 1 part ammonia to 30 parts alcohol; then 2 to 4 hours in 2 changes of absolute alcohol. Clear in chloroform or cedar oil, and embed in paraffin. Leave sections 12 hours or more in 95% alcohol, containing 2% ammonia, then rinse in 80% alcohol. Transfer to 40% silver nitrate in the dark for 20 minutes, several hours, or days. Rinse in distilled water. Cover sections with 20% formalin for 2 to 5 minutes and drain without washing. Cover with diammoniacal silver solution heated to 30 to 35° (prepared by adding ammonia to 4 cc. of 20% $AgNO_3$ until precipitate forms and is redissolved, then 2 drops in excess, after which 4 cc. distilled water is added.) Allow this to remain on slides until sections turn a yellowish brown, or until nerve fibers have been stained. Rinse in distilled water. Tone 10 to 15 minutes with 0.33% gold chloride containing 10 drops of glacial acetic to every 50 cc. Fix in 5% sodium hyposulfite for about 5 minutes. Wash in several changes of water or under tap. Dehydrate, clear and mount. Three alternate methods are also recommended. All four methods stain nervous elements black, including fibrillae and terminations. If a counterstain is desired, it may be added before final dehydration.

RUYTER, J. H. C. Eine einfache Methode für das Aufkleben von Zelloidin-Paraffinschnitten. Zts. Wis. Mikr., **48**, 226–227. 1931.

The original celloidin paraffin method of Apathy has been improved by Mayer and Peterfi by the use of methyl benzoate. Jordan, v. Möhlendorff, and Tschernyachinsky's modification made a higher percentage of impregnated celloidin possible. This

facilitated cutting and better sections could be obtained, but this high content of celloidin made mounting rather difficult, for such sections would spread poorly on the slides.

The author offers an acetone-methyl-benzoate medium, instead of water, for spreading sections on the slides. The latter softens the celloidin, without absolutely dissolving it. Prepare as follows: mix 2 cc. of acetone with 1 drop of methyl benzoate, and add 8 cc. of distilled water. Add 2 to 3 drops of albumen-glycerin if desired. Mount as in the usual paraffin water procedure, but use less heat, for the sections begin to straighten even before any heat is applied.

SIMPSON, W. M. and KREAGER, H. H. A rapid inexpensive method for preparing permanent tissue sections. J. Lab. & Clin. Med., **16**, 611–617. 1931.

A detailed account of fixation, arrangement of specimen of one case on a string under given numbers, ordering, handling, embedding and staining is given. It is a time-saving method, which enables the technician to offer slides the day following the operation. Great quantities of material may be handled simultaneously. Absolute alcohol is entirely dispensed with.

TURNER, J. P. A simple apparatus for washing Protozoa. Science, **74**, 99–100. 1931.

This is a simplified and very satisfactory modification of Caullery's and Chapellier's method for washing protozoa. Get a section of glass tubing, 8 cm. long and 5 to 6 mm. inside diameter. Heat at one or both ends and spread slightly after the manner of the bulb of a pipette. Fasten tightly a piece of bolting cloth over the enlarged end of the tube by means of a rubber band. No. 20 silk bolting cloth is satisfactory for average sized protozoa, No. 25 for smaller forms. This may be unsuitable for the very small ones. Place the tube, covered end down, in a small bottle, and with the mouth end well up.

Transfer organisms to tube. As soon as the fluid drains off let a small stream of water into the tube. This provides a steady flow of fresh water as the column of water between the mouth of the bottle and the upper end of the tube exerts just enough pressure to cause a gentle flow thru the tube. The waste escapes thru the bottle. Should the running water contain much foreign matter use bolting cloth of fine mesh before allowing it to pass thru the tube.

After washing the material may be handled as usual. If preferred it may be stained and dehydrated before removing it from the tube by merely pipetting in the fluids, or by transferring the tube with the organisms from one bottle to another with the necessary reagents.

VON GELEI, J. and HORVATH, P. Eine nasse Silber- bzw. Goldmethode für die Herstellung der reizleitenden Elemente bei den Ciliaten. Zts. Wis. Mikr., **48**, 9–29. 1931.

By means of the proposed method, subpellicular, conductive and other structural elements of the protozoa are brought out. The osmium toluidin method of Gelei shows basal bodies of cilia and trichocytes, and the connection between them, but does not bring out various details. The silver method of Klein, in which the organisms are dried on the slide without fixation, brings out various structures, but has the disadvantage of exposing the protozoa in one plane.

For the wet silver or gold method proceed as follows: 1) fix in fresh formol sublimate, (5 cc. formalin to 95 cc. of concentrated $HgCl_2$) 1 to 3 minutes. 2) Centrifuge and wash in two changes of tap or river water. 3) Silver bath (1 to 2% $AgNO_3$) for 2 to 30 minutes. 4) Centrifuge and reduce in direct sunlight, in distilled water, or if staining of the cilia is desired, in tap water; or equal parts of 1/10 formol and 1/10 ammonia may be used. 5) Wash in several changes of distilled water. 6) Run thru the alcohols. 7) Mount in glycerin alcohol, or pass thru xylol to xylol balsam. Glycerin alone may be used, with omission of step 6 (dehydration). 8) If gold impregnation is desired, introduce this treatment at step 5. For the gold bath use 3 cc. of distilled water to 1 drop of 1% gold chloride; apply for one to several hours.

Agitation of the tubes with every new fluid is recommended, and pouring off the fluid after centrifuging, instead of pipetting. Good results were thus obtained with *Paramecium, Colpedium, Euplotes* and *Vorticella*. This method is not as permanent as Klein's for the material fades out easily. This and Klein's method supplement each other in the authors' opinion.